IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Wu et al. Confirmation No: 3823

Serial No.: 10/576,491 Group Art Unit: 1652

Filed: April 18, 2006 Examiner: S. Swope

For: Protease with Improved Stability in Detergents

DECLARATION OF DR. JÜRGEN KNÖTZEL UNDER 37 CFR 1.132

Commissioner for Patents
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Sir:

I, Jürgen Knötzel, do hereby state and declare as follows:

1. I obtained a Diplom (equivalent to the Masters degree) in Biology in 1984 and a Doctor of Sciences (Dr. rer. nat.) in 1988 from University of Bremen, Germany. In 1998, I obtained a Habilitation and Venia legendi in Cell and Molecular Biology (Dr. rer. nat. habil.), also from University of Bremen, Germany. From 1985-1988, I was a Research Assistant at University of Bremen, Germany. From 1988-1992, I was a Postdoctoral Fellow at the Department of Physiology, Carlsberg Laboratory, Copenhagen, Denmark. From 1992-1998, I was a University assistant (C1) at the Institute of Cell Biology, Biochemistry and Biotechnology, University of Bremen. From 1999-2000, I was Associate Research Professor at the Department of Plant Biology, Laboratory of Plant Biochemistry, The Royal Veterinary and Agricultural University, Frederiksberg, Copenhagen, Denmark. In 2001, I joined Novozymes A/S, Bagsværd, Denmark, the assignee of the above-identified application as a Research Scientist, Detergent Applications I. Since 2005, I have been a Science Manager, Detergent Applications I, with Novozymes A/S. At Novozymes A/S, I work on developing enzymes and particularly protease enzymes for use in detergents.

2. I have read and understood the above-identified patent application and Isono et al., US Patent No. 3,655,670 ("Isono").

3. The following experiments were carried out under my direction and supervision.

4. The following materials were used:

Enzymes:

Trypsin *Fusarium solani* of SEQ ID NO: 2 (14,28mg/ml) (MW: 22704)

Porcine Trypsin (protease control) (9,5mg/ml)

Chemicals:

TRIS-base

NaOH

CHES

HEPES

Protazyme AK tablets

Incubation buffer: 0.5M TRIS-HCl pH 11

Substrate buffer: 1 tablet Protazym AK to 4 mL CHES-HEPES buffer

5. LAS-detergent (Detergent 1) was prepared according to Table 1 of Isono as follows:

25% LAS: 27,47g/100ml

40% Sodium tri-phosphate: 40g/100ml

29% Sodium sulphate: 29g/100ml

5% Sodium silicate: 5g/100ml

1% Carboxymethyl cellulose (Finnfix BDA): 1g/100ml

Stock made 10 times diluted, and the stock is further diluted before use according to Isono et al.: 500mg LAS-detergent in 20 mL 0.05M TRIS-HCl pH 11.

6. Protease activity of Trypsin *Fusarium solani* of SEQ ID NO: 2 and of a control protease was evaluated in the presence of TRIS pH 11 buffer or in LAS-detergent prepared according to Isono as follows:

Incubation:

- Dilute proteases in 1mL 0.05M TRIS pH 11 buffer /or in LAS-detergent pH 11
- Mix on thermomixer for 20 min /37°C

Protease activity:

- 20µl incubation sample + 1000µl Protazym AK. (Blank: 20µl buffer).
- Mix on thermomixer for 15 min /37°C.
- Add 100µl 1M NaOH.
- Centrifuge 3 min /15'000 xg.
- 200µl supernatant transferred to micro well plate. Measure absorption at 650nm.

The control trypsin protease was selected because it is from the same family (S1A) as the *F. solani* trypsin protease and is known to have wash performance in LAS-containing detergents.

It is not clear at which concentrations the proteases are measured in Isono. The control trypsin protease was used to determine the concentrations at which a trypsin protease would work in the Isono assay.

7. The results of protease activity are shown in Tables 1-4.

Table 1. Porcine Trypsin (Control) in 0.05M Tris buffer

Concentration µg /ml	µl Porcine Trypsin in 1 mL TRIS pH 11	µg in assay (20µl)	Abs.650nm
10	1,1	0,2	0,159
25	2,63	0,5	0,446
40	4,21	0,8	1,334
50	5,26	1	2,079

Table 2. Porcine Trypsin (Control) in LAS-Detergent

Concentration µg/ml	µl Porcine in 1 mL LAS Detergent	µg in assay (20µl)	Abs.650nm
1	1,1 (1:10)	0,02	-0,001
5	5,26 (1:10)	0,1	-0,012
10	1,1	0,2	0,148
20	2,1	0,4	0,440
40	4,21	0,8	1,046

Table 3. *Fusarium solani* trypsin in 0.05M Tris buffer

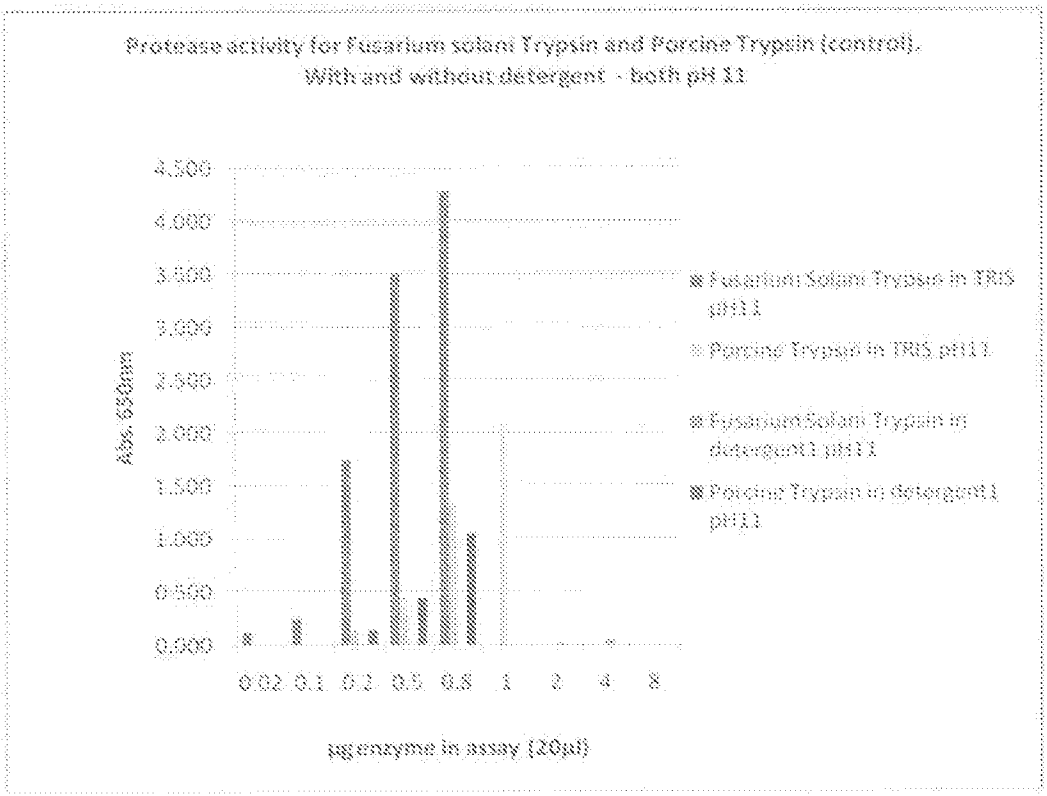
Concentration µg/ml	µl Fusarium solani (1:100) in 1 mL TRIS pH 11	µg in assay (20µl)	Abs.650nm
1,0	7,0	0,02	0,121
5	35	0,1	0,250
10	70	0,2	1,743
20	140	0,4	3,507
40	280	0,8	4,283

Table 4. *Fusarium solani* trypsin in LAS-Detergent

Concentration µg/ml.	µl Fusarium solani (1:100 / 1:10) in 1 ml. Detergent 1 pH 11	µg in assay (20µl)	Abs.650nm
1,0	7,0 (1:100)	0,02	-0,009
5,0	35 (1:100)	0,1	-0,012
10	70 (1:100)	0,2	-0,018
20	140 (1:100)	0,4	-0,020
40	280 (1:100)	0,8	-0,015
10	7,0 (1:10)	0,2	0,015
50	35 (1:10)	1,0	-0,006
100	70 (1:10)	2,0	0,011
200	140 (1:10)	4,0	0,052
400	280 (1:10)	8,0	0,009

These data are represented graphically in Figure 1.

Figure 1.



8. As can be seen from Tables 3-4 and Figure 1, Trypsin *Fusarium solani* of the patent application in LAS-detergent showed no activity when tested at the same concentrations as those that showed a high level of activity in 0.05 M Tris-buffer. Even at a 10-fold concentration increase of *F. solani* trypsin, essentially no activity was seen in the presence of LAS-detergent.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 21 day of January, 2011

Jürgen Kruszel